Assessment of the physiological impact of endocrine disrupting chemicals on salmonid fish from selected sites in Scotland and Northern Ireland

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EXECUTIVE SUMMARY

1. Recent research in the UK and elsewhere has demonstrated that industrial and domestic effluents may contain compounds of both natural and synthetic origin which are steroid estrogens, or mimic the activity of steroid estrogens. Although present in solution at very low concentrations the sensitivity of the endocrine system of animals to interference is such that these contaminants represent a potentially disruptive threat.

2. A survey of gonadal structure in roach populations in English rivers revealed that a high proportion (25% – 60%) of male fish within the sampled populations displayed evidence of abnormal gonadal development in the form of ovarian tissue within the testes. These effects were most pronounced at sites where discharged effluents represented a high proportion of total flow in the river. The functional significance of these observations has yet to be established.

3. Concerns have been raised regarding the possible susceptibility of valuable salmon and trout populations to endocrine disruptive processes. This report describes the results of a field-based investigation of indicators of estrogen exposure in salmonid fish exposed to potentially estrogenic effluent discharges at sites in Scotland and Northern Ireland.

4. This study addressed the possibility that native salmonid populations are being exposed to estrogenic contaminants via both domestic and industrial discharges and that this exposure may result in inappropriate and possibly detrimental physiological effects in exposed fish.

5. This objective was addressed by the sampling of brown trout and Atlantic salmon from sites receiving a STW discharge and from pristine sites. Blood levels of the estrogen-inducible yolk precursor vitellogenin were measured to assess whether there was evidence that the targeted populations were exposed to acute estrogen exposure. In addition, gonadal material sampled from each fish was examined microscopically for evidence of abnormal reproductive development, the presence of which would be indicative of early or possibly chronic exposure to estrogens.

6. A total of 209 fish were examined from four sites in Scotland [R. Teviot, Slitrig Water

(tributary of the R. Teviot), Wilson Burn and Ale Water (pristine sites)]. Fish sampled from the R. Teviot were removed downstream of the Hawick STW discharge. In all the fish which were sampled blood vitellogenin levels were low, displaying no evidence of environmental estrogen-induced elevation. The gonadal structure of all the sampled fish was normal, with no evidence of ovarian tissue in male testes and no evidence of unusual ovarian development in female fish.

7. A total of 64 fish were examined from three sites in Northern Ireland (Minnowburn, Collin River and Ravernet River; all tributaries of the R. Lagan). As was the case for the Scottish sites, no evidence of gonadal abnormalities was obtained in fish from any site.

8. In addition to the field sampling, the estrogenicity of the effluents discharged at each site were evaluated by bioassay. In Northern Ireland, rainbow trout were held in cages submerged in the vicinity of the New Holland Sewage Treatment Works (STW) effluent discharge (R. Lagan). An aquarium-based exposure of rainbow trout to Hawick STW (R. Teviot) final effluent, at concentrations up to 100%, was employed. Analysis of blood vitellogenin levels after a period of or 11 – 14 days exposure in aquaria revealed that the Hawick STW effluent was not estrogenic. However, fish exposed to New Holland STW final effluent *in situ* for 3 weeks displayed elevated blood vitellogenin levels, relative to fish caged at control sites, indicating that the effluent contained estrogenic components.

9. Water samples collected from both study sites were analysed for total alkylphenols and alkylphenol ethoxylates (APEs; ubiquitous environmental contaminants and known estrogen mimics found in high concentrations in English rivers with a high incidence of intersexuality). Samples from the Hawick sites contained very low levels of APEs (< 3 :g l^{-1}). Similar levels were found in samples from the sites in Northern Ireland with the exception of effluent samples taken downstream of the Barbour Campbell Linen Mill which contained 12 :g l^{-1} total APEs. Steroids were not quantified in these samples.

10. The microscopic examination of gonad structure, and analysis of blood vitellogenin levels, indicated that there was no evidence of exposure to estrogenic contaminants in the salmonid fish populations of the R. Teviot or R. Lagan.

11. A number of factors probably contribute to this result. Salmonid fish exhibit a life-history

very different to that of cyprinid fish such as roach in which effluent-related intersexuality has been reported. Trout and salmon normally spawn in clean gravel beds in the upper reaches of a river system and newly-hatched and early juvenile salmonid fish remain in these relatively pristine stretches of river. They are therefore not usually exposed to point-source effluent discharges during critical early developmental stages when the reproductive system is particularly sensitive to external influences. In addition, both salmon and migratory brown trout (sea trout) are anadromous fish, spending a significant proportion of their lives at sea. These factors contrast with the potential for life-long exposure to contaminants displayed by non-migratory cyprinid fish which spawn in the main body of the river. Additionally, salmonid fish are less tolerant of poor water quality than cyprinid fish and even the non-migratory brown trout are therefore not normally present in rivers subject to high effluent input. Most rivers which support populations of salmonid fish do not pass through heavily populated or industrialised areas. There is therefore less likelihood that these fish populations will encounter high concentrations of endocrine disrupting chemicals (EDCs).

12. Overall, these data do not provide absolute reassurance that salmonid fish are unaffected by estrogens and estrogen mimics present in effluent discharges but they do indicate that if a problem does exist it may be localised and on a smaller scale than has been reported for cyprinid fish.

13. Consideration should be given to a more widespread survey of gonad structure in salmonid fish populations in order to confirm these preliminary conclusions. It should also be considered whether reproductive performance of salmonid fish might be influenced by exposure to EDCs via mechanisms which do not involve alterations in gonadal structure (e.g. alterations in fecundity, gamete quality, behavioural changes). In addition, the possibility that aspects of the salmonid life-history other than reproductive development may be affected by EDCs should be examined.

14. For example, smoltification (pre-migratory sea water adaptation) is a critically important aspect of the salmonid life history. Smoltification is closely regulated by the endocrine system and may be susceptible to interference by chemicals which mimic or interfere with the activity of hormones other than estrogens.

15. It should also be considered whether sources of contamination other than effluent

discharges (e.g. agricultural run-off) may expose salmonid fish to endocrine-disrupting chemicals.

KEY WORDS

Endocrine disruption, trout, salmon, reproduction, vitellogenin, gonadal structure, effluent, sewage, R. Tweed, R. Teviot, R. Lagan.

1. INTRODUCTION

1.1 Background - What is endocrine disruption?

All animals, both vertebrate and invertebrate, utilise internal chemical signalling mechanisms to co-ordinate and control growth, development, metabolism, reproduction and behaviour. This signalling infrastructure is known as the endocrine system and the constituent chemical messengers as hormones. In vertebrates the endocrine system is well-characterised whereas in invertebrates the endocrine system of only two major groups (insects and crustaceans) enjoys a similar level of understanding.

During the last decade there has been increasing concern as evidence has accumulated that many chemicals entering the environment, and to which both humans and wildlife are exposed, may be capable of interfering in the normal function of the endocrine system (Colborn *et al*., 1993; Colborn, 1995; Hose & Guillette, 1995; Toppari *et al*., 1996). There is as yet no firm evidence for endocrine-disruptive effects within the human population (Safe, 2000) whereas a number of well-documented cases exist for effects on wildlife (Vos *et al*., 2000).

The potential for adverse effects arises because some contaminants are capable of mimicking the action of endogenous (non-peptide) hormones. Hormones exert effects by interacting with a specific receptor and some contaminant chemicals possess structural characteristics which allow them also to interact with the same receptor sites (Tyler *et al*., 1998). Inappropriate and anomalous physiological effects can thus arise due to stimulation of or interference with normal endocrine events by chemicals of exogenous origin.

Because the endocrine system is sensitive to hormone signals which are present in the blood or body fluids at very low concentrations (pg ml^{-1} - ng ml^{-1}) contaminant-induced effects can occur at relatively low concentrations of exposure, certainly below the levels required for detection of conventional toxicity end-points such as the LC_{50}. In addition, because the effects of exposure to EDCs are sub-lethal and relatively subtle most EDCs were not identified as such during routine regulatory testing.

Two major issues are currently being addressed by researchers.

- To what extent is the human population being exposed to endocrine disrupting chemicals and what effects are occurring as a consequence of any such exposure?
- To what extent is wildlife at risk from endocrine disrupting chemicals?

1.2 Endocrine disruption in the freshwater environment

There is concern in both Europe (Tyler & Routledge, 1998) and North America (Guillette & Guillette, 1996) that contaminants entering the aquatic environment may be capable of exerting endocrine disrupting effects on aquatic biota. The range of suspected EDCs encompasses natural hormones (e.g. 17β-estradiol), synthetic hormones (e.g diethylstilbestrol, DES), phytoestrogens (e.g. genistein), pesticides (e.g. DDT), polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (e.g. TCDD), alkylphenol and alkylphenolethoxylates (e.g nonylphenol, octylphenol), phthalate esters (e.g. benzylbutylphthalate), bisphenol A, organotin compounds (e.g. tributyltin, TBT) (Miyamoto & Klein, 1998).

A considerable body of evidence is available which demonstrates hormone-like activity of these and other classes of compounds under laboratory conditions both *in vitro* and *in vivo* (Tyler *et al*., 1998). However, firm evidence for effects on animals in the natural environment is more limited and recent commentaries have highlighted the need for epidemiological and wildlife studies to fully evaluate the extent of the problem (Ashby *et al*., 1997; Taylor & Harrison, 1999). In the UK, evidence for effects of EDCs on free-living animals is restricted to reports of adverse changes in the reproductive system of molluscs exposed to TBT (Matthiessen & Gibbs, 1998) and in fish exposed to sewage treatment works (STW) effluent (Tyler & Routledge, 1998).

1.2.1 Reproductive abnormalities in fish exposed to STW effluents

In the UK, interest in EDCs in the freshwater aquatic environment has focused on the effects of estrogens and estrogen mimics present in STW effluent. This emphasis on the reproductive

system and feminizing influence of estrogens in particular has arisen because early observations on hermaphroditism in roach (*Rutilus rutilus*), and of anomalous yolk protein precursor (vitellogenin; VTG) levels in the blood of male rainbow trout, were indicative of estrogen-mediated effects.

Induction of VTG is an estrogen-dependent process and, although VTG is not normally synthesised by males (Copeland *et al*., 1986), male fish possess the hepatic estrogen receptor (Pottinger, 1986; Pakdel *et al.*, 1997) and VTG gene (Le Guellec *et al*., 1988). The normally low levels of VTG in the blood of male fish therefore make them sensitive bioindicators of estrogen exposure (Purdom *et al*., 1994). [It should again be emphasised that the phenomenon of endocrine disruption need not be restricted to estrogenic mimics, or free estrogens, only. In theory, any receptor-mediated endocrine process is potentially susceptible to interference.] Direct measurement of blood VTG levels in fish by either radioimmunoassay (RIA; Harries *et al*., 1999), enzyme-linked immunosorbent assay (ELISA; Folmar *et al*., 1996) or SDS-PAGE / Western blotting (Orlando *et al*., 1999) has been successful in detecting the exposure of fish to estrogens and estrogen-mimics in both field situations (Harries *et al*., 1997; Orlando *et al*., 1999) and under laboratory conditions (Korsgaard & Pedersen, 1998; Panter *et al*., 1998; Gronen *et al*., 1999). Detection of VTG gene expression via semi-quantitative measurement of levels of specific mRNA has also been utilised as an estrogen-exposure marker in a number of species including rainbow trout (Flouriot *et al*., 1995) and schelly (*Coregonus lavaretus*; Mellanen *et al*., 1999) although there may not be a direct correlation between VTG mRNA induction and VTG production (Gagnð & Blaise, 1998).

It was inferred that the gonadal abnormalities reported in roach were related to effluent from nearby STWs (Tyler & Routledge, 1998). Subsequent studies demonstrated the induction of VTG in caged male trout exposed to STW effluent (Purdom *et al*., 1994). Elevation of blood VTG levels has been demonstrated to occur in fish caged at distances of up to 5 km downstream from the effluent input (Harries *et al*., 1997) providing clear evidence that STW effluent can contain an estrogenic component(s).

Fractionation of STW effluent has indicated that the natural steroids 17β-estradiol and estrone, of human or animal origin, were primarily responsible for the estrogenicity of the effluent (Desbrow *et al*., 1998). Although these steroids are excreted as hormonally inactive conjugates, microbial activity is evidently capable of biotransforming the inert conjugates into

the active steroid (Panter *et al*., 1999). Whether this transformation occurs within the STW, or prior to treatment is presently unclear. The contribution of other potentially estrogenic contaminants, such as alkylphenols (Jobling & Sumpter, 1993), cannot be excluded but is more likely to be significant in areas where industrial discharges occur.

Following the revelation that STW effluents may contain significant quantities of estrogenic material, and that this was potentially capable of interfering with normal reproductive processes in fish, a survey of gonadal structure in wild populations of roach in England and Eire was carried out. This exercise revealed there to be a high incidence of abnormal gonadal structure in fish captured downstream of STWs (Jobling *et al*., 1998). Microscopic examination of gonadal tissue from ostensibly male fish in some cases revealed the presence of cell types normally characteristic of female gonads. This "intersex" condition was present in a high proportion of fish captured downstream of STWs (up to 100%) while the maximal occurrence of intersexuality at upstream sites was 44%. These figures contrast with a maximum of 18% intersex fish at control sites (Jobling *et al*., 1998). The authors of that study concluded that the concentration of sewage effluent in rivers is a major causal factor in the presence of intersexuality in fish.

Evidence for the estrogenic effects of STW effluent is not restricted to the UK. Similar observations have been made in the US (Folmar *et al*., 1996), Norway (Knudsen *et al*., 1997) and Australia (Batty & Lim, 1999).

There is as yet no clear evidence for population level effects of inappropriate VTG production and intersexuality arising from exposure to STW effluent.

1.2.2 The Scotland and Northern Ireland perspective

The UK rivers which have been studied for evidence of endocrine disruption among fish have been to date characteristically slow-moving lowland rivers populated predominantly by cyprinid fish. These are non-migratory species (e.g. roach, chub, dace) whose entire life history is conducted in the main body of the river. Therefore, where contaminants are present, it is theoretically possible for individual fish to receive life–long exposure to contaminants from egg and early development through to spawning adult.

In Scotland and Northern Ireland, salmonid fish represent a more significant and economically important fisheries resource than cyprinid fish. Salmonid fish exhibit more complex life-histories than cyprinids. All Atlantic salmon (*Salmo salar*) and a proportion of brown trout (*Salmo trutta*) are anadramous, migrating to sea as smolts and returning to freshwater to spawn in the headwaters of the rivers in which they were themselves juveniles. All salmonid fish have a requirement for clean unpolluted water and inhabit (with some exceptions) fast-flowing rivers or large lakes with sufficient depth to avoid warm summer temperatures.

Spawning occurs in upland streams typified by fast flow and fine gravel beds. After the fish hatch and leave the gravel beds they become territorial, in contrast with the gregarious shoaling cyprinid species. After 1 or 2 years in freshwater, those individuals which are destined to migrate seawards, undergoing a process of physiological adaptation to seawater known as smoltification. One or more years are spent feeding at sea before the fish return to spawn in the same river in which they hatched. Mature male and female fish congregate on the spawning sites and spawning ensues. Surviving adults may return to sea and repeat the process over a number of seasons.

Clearly, for salmonid fish, the risk of exposure to contaminants from point-source effluent inputs is limited to those periods of their life history during which they may be resident in stretches of river passing through conurbations. During their early developmental stages, and in the period of pre-spawning maturation, considered to be the stages most sensitive and vulnerable to the effects of endocrine-disrupting chemicals, salmonid fish are unlikely to be exposed to potential EDCs.

Although salmonid fish, particularly rainbow trout, have been widely used as a model species in the assessment of EDCs and effluents there has been no systematic study of natural populations of salmonids to determine whether these species are affected by STW discharges.

1.3 Study Aims

This study was designed to address the possibility that native salmonid populations in Scotland and Northern Ireland are being exposed to estrogenic contaminants via domestic and/or industrial discharges and that this exposure is resulting in adverse effects. Evidence of

inappropriate exposure to estrogens was sought by carrying out a field-based investigation in which physiological and histological endpoints were assessed in salmonid fish at both impacted and unimpacted sites.

Estrogen exposure was evaluated by (i) determination of blood VTG levels and (ii) examination of the gonadal structure of fish caught from sites upstream and downstream of STW inputs and from unimpacted sites.

2. METHODS

2.1 Selection of sites

2.1.1 Scotland

Discussion with local SEPA staff identified the most appropriate impacted site for the purposes of this study as being on the River Teviot at Hawick, Roxburghshire. Effluent from the town sewage treatment works (STW) is discharged into the river and the town has a number of textile factories, whose wastes are also discharged through the STW. It was confirmed by SEPA that at least two companies in the town use chemicals known to be estrogenic.

Hawick STW serves a population of approximately 15,000 although its population equivalent is 28,000. Waste water receives full treatment with secondary treatment by activated sludge using a diffused air system. The mean rate of discharge into the river (measured during the period Aug 1996 – Oct 1997) was 74.3 l sec^{-1} (maximum 180 l sec^{-1} – minimum 17 l sec^{-1}).

The R. Teviot flows west to east through the centre of Hawick. Fish were sampled from the stretch immediately downstream of the STW input (grid reference NT517155). A site upstream of the STW and south of Hawick, on the Slitrig Water which joins the R. Teviot in the centre of Hawick, was also selected for sampling (grid reference NT504127). Two additional sites were selected to provide fish from a "pristine" environment. These were on the Wilson Burn (grid reference NT386149) and Ale Water (grid reference NT384145), both of which drain into Alemoor Reservoir, west of Hawick.

2.1.2 Northern Ireland

The River Lagan, which enters the Irish Sea through Belfast, drains a catchment of 550 Km^2 which is essentially agricultural in its upper part and includes Belfast and Lisburn in the lower part. The catchment contains a human population of approximately 600,000 people. While much of Belfast's STW effluent enters the estuarine or marine environment at the mouth of the river, the freshwater reaches receive treated effluents from at least 100,000 people, mainly

from the town of Lisburn (population 80,000). Effluent from New Holland STW, the largest and most modern works discharging to the freshwater portion of the river, may constitute 50% of the river flow at low flow conditions at the point of entering the river. Outside of flood flows and extreme low flow periods, ratios of STW flow to river flow are of the order of 1:3 to 1:20. Mean inflow (and hence outflow) to New Holland STW over the period of the study was 257 l sec^{-1}, (max 523 l sec^{-1}, min 211 l sec^{-1}).

That salmonids exist in the Lagan may seem surprising, but the river has been the site of a successful salmon reintroduction programme since 1991, and salmon, brown trout and sea trout breed in several tributaries entering the main river downstream of the STW and in the main river upstream of the major STWs.

Northern Ireland has a very restricted fish fauna, and has no chub, dace, barbel or grayling, all species which might otherwise compete with salmonids in fast or moderately flowing lowland streams. Hence many lowland streams contain brown trout as the most important species in areas where other equivalent rivers in other parts of the UK would have coarse fisheries.

The Lagan is an ideal candidate for a study of endocrine disruption in salmonids, as there are: a developing salmonid fishery with readily available samples of wild brown trout, a STW feeding the freshwater reaches of the river with high population loading, and several options on restricted public access sites for caged fish experimentation, including not least the final filtration tanks of the STW at New Holland, Lisburn.

The River Bush in County Antrim offers an ideal control site, having a long history of salmonid study at the River Bush Salmon Station at Bushmills and an almost entirely agricultural (mainly pastureland) catchment of similar size to that of the Lagan. The small town of Bushmills, (population circa 5000) whose STW enters downstream of the Salmon Station, is the only a significant human sewage source. The Salmon Station itself offers ample opportunity to place caged fish experiments in a readily controlled flow of clean raw river water.

2.2 Assessment of effluent estrogenicity

2.2.1 Use of caged trout as biomonitors of estrogen exposure

2.2.1.1 Scotland

On 1.10.98 polythene mesh cages (100 x 50 x 50 cm) were installed on the R. Teviot. The cages were placed in pairs upstream, adjacent to, and downstream of the STW effluent. Each cage was populated with eight immature all-female rainbow trout (*Oncorhynchus mykiss* Walbaum; supplied by Gala Fish Farm). Each fish was dye-marked (alcian blue) by needleless injector (Panjet; Wright Dental) and blood-sampled prior to transfer to the cage. The cages were moored to steel angle posts embedded into the river bed. A further six cages were sited on the Slitrig Water to provide a control comparison. A major concern at the time of installation, for the R. Teviot cages, was the possibility of high water or flood conditions. Therefore the cages were sited with regard to retrieval, and taking into consideration the likely effects of high flows.

The Slitrig cages were readily accessible by wading because of the relatively low level of water in the Slitrig. However, the cages moored in the Teviot, although visible from the bankside, were moored in deeper water and were only accessible using chest waders. Previous experience with this cage system on rivers in Dorset suggested that the equipment would not be tampered with during the exposure period.

The cages were checked daily and remained in place throughout the first week. However, during the second week cages were removed from the two downstream sites on the R. Teviot and all cages on the Slitrig Water were opened and the fish were removed. Only the two upstream cages on the R. Teviot survived intact. From these, all sixteen fish were retrieved alive and in good condition.

We conclude that any future caging study carried out at sites with public access will require cages with a more secure design and more limited portability than those employed in this study.

2.2.1.2 Northern Ireland

A total of four test sites on the River Lagan and one control site on the River Bush were chosen for this study. Abnormally high water levels several days after the initial deployment led to difficulties in anchoring cages securely at two of the downstream sites: the point of entry of New Holland STW waste stream and at Stranmillis fish trap. As result all fish were lost from these two sites before the mid-deployment sampling date. The test and control site locations for which a full experiment was completed are shown in Table 1. In addition to the in-river experiments, on 19/1/00 two additional cages of fish were placed directly in the final effluent of New Holland STW, in the tertiary treatment (microstrainer) tanks downstream of the strainer drums. These replaced the cages lost from the effluent stream where it enters the river.

Table 1. Experimental sites, dates of sampling, and survival of caged fish, Northern Ireland.

Test Sites: Lagan River	**Deployment and initial blood sample (No. survivors)**	**Mid-experiment blood sample (No. survivors)**	**Termination and final blood sample (No. survivors)**
Barbour Campbell Threads Linen Mill (Upstream of STW discharge) [IGR J283654]	1/11/99 (20)	17/11/99 (20)	24/11/99 (20)
New Holland STW, Hilden (Final Effluent tanks) [IGR J284654]	19/01/00 (20)	1/2/00 (18)	9/2/00 (16)
Lambeg Fish Pass [IGR J279661]	1/11/99 (20)	17/11/99 (19)	24/11/99 (18)
Control Site			
DANI River Bush Salmon Station [IGR J940405]	28/10/99 (20)	10/11/99 (20)	23/11/99 (20)

Each cage was constructed from a galvanised steel “dexion” frame of 0.5 x 0.5 x 1.0 m covered with 13 mm square knotless mesh sewn into a box fitting tightly on the frames. A detachable mesh lid allowed access to the cages by unstitching one top edge. The fish used were monosex female immature stock from the DANI trout rearing farm at Movanagher on the River Bann, Co. Antrim. The all female immature status of the fish was confirmed by histological examination of the gonads. Ten fish of 15 - 20 cm long (approx) were used per

cage, with each fish uniquely indentified by panjet (Wright Dental Group) dye-marking through rays in one or two fins. Each fish was weighed, measured (fork length) and blood sampled from the caudal vessels prior to transfer to the cages. Cages were checked daily and any dead or moribund fish were removed. Only the fish held in the tertiary treatment tanks at the STW were fed with pellets during the experiments, due to the absence of any natural food items in the water at this site. Stomachs of the fish held in cages in open river situations all contained the remains of natural food items when examined at the termination of the experiments.

2.2.2 Semi-static aquarium exposure of rainbow trout to STW effluent

2.2.2.1 Scotland

Following the failure of the caging exercise, and after discussion with the Project Manager, the estrogenicity of the Hawick STW effluent was evaluated using a semi-static aquarium exposure system.

Effluent exposure experiment 1: On 17.11.98 six rainbow trout (mixed-sex, 2-years old, Stirling strain) were transferred from outdoor holding tanks to each of eight glass aquaria (90 x 40 x 30 cm; 80 litres) sited in the CEH indoor experimental facility. Each tank contained lake water which was continuously aerated via a pump and airstone. Prior to transfer, each fish within a group of six was anaesthetized (2-phenoxyethanol, 1:2000), dye-marked for identification using a needleless injector (Alcian blue; Panjet, Wright Dental Group), and a blood sample (<1.0 ml) was removed from the Cuverian sinus into a heparinized syringe. The blood samples were transferred to capped polypropylene tubes containing an anti-protease (aprotinin; 2 TIU ml^{-1} blood in 10 µl distilled water), mixed by inversion, and stored on ice (< 2 h) until being centrifuged at 4^{o}C. Plasma was removed from the pelleted cells by pipette, transferred to a labelled capped polypropylene tube, and frozen (-20^{o}C) until required for assay.

On 24.11.98, one week after transferring the fish to the aquaria, effluent was collected from the Hawick STW and transported to CEH Windermere in 25 litre polypropylene containers. Where appropriate, lake water was removed from the aquaria and effluent was added to give final concentrations of effluent in 80 litres of 0% (control), 7.5%, 15%, and 30%, in duplicate. During the transfer of effluent to the tanks fish were temporarily placed in a large bucket

containing lake water.

The effluent was replenished on days 4 and 8 and on day 11 the study was terminated. The fish from each tank were netted into anaesthetic, identified by dye-mark and blood sampled. Blood samples were treated as described above. The fish were killed by a blow to the head and weighed, measured and sexed.

Effluent exposure experiment 2: The results of the first study were negative. In order to confirm these results, and to establish whether the failure to observe a vitellogenic response in the exposed fish was due to the high dilution of effluent, a second study was carried out during May 1999. On 10.5.99 final effluent was collected from the Hawick STW and transported to CEH Windermere in 25 litre polypropylene containers. Effluent and lake water were added to glass aquaria (90 x 40 x 30 cm; 80 litres) to give final concentrations of effluent in duplicate of 0% (control), 33%, 66%, and 100%. Each tank was continuously aerated via a pump and airstone. On 11.5.99 four rainbow trout (mixed-sex, 2-years old, Stirling strain) were transferred from outdoor holding tanks to each of the eight glass aquaria. Prior to transfer, each fish within a group of four was anaesthetized and marked as for the previous exposure experiment. Blood samples were collected as described above and plasma was separated and stored frozen.

Further batches of effluent were collected from Hawick STW on days 2, 4, 7, 9, and 11. These were used to completely replenish the contents of each tank. During the transfer of effluent to the tanks fish were temporarily placed in a large bucket containing lake water. On day 14 the study was terminated. The fish from each tank were netted into anaesthetic, identified by dye-mark and blood sampled. Blood samples were treated as described above. The fish were killed by a blow to the head and weighed, measured and sexed.

2.3 Field sampling methods

2.3.1 Scotland

On three occasions (18/19.11.98; 21.4.99; 6/7.9.99) stretches of river at each study site (R. Teviot and Slitrig Water together with either Ale Water or Wilson Burn) were electrofished to

obtain representative samples of the native salmonid population. Details of the sampling programme are provided in Table 2.

Table 2. Summary of sampling programme, Scotland.

Site	Date	Number of fish sampled	
		Male	Female
R. Teviot (NT517155)	18-19/11/98	6	11
Slitrig water (NT504127)	18-19/11/98	13	13
Wilson Burn (NT386149)	18-19/11/98	11	9
R. Teviot	29/4/99	12	8
Slitrig water	21/4/99	13	6
Wilson Burn	21/4/99	9	11
R. Teviot	6-7/9/99	16	14
Slitrig water	6-7/9/99	18	12
Ale water (NT384145)	6-7/9/99	20	10
Totals		119	93

Immediately after capture, fish were transferred to anaesthetic (2-phenoxyethanol; 1:2000). When fully anaesthetised, a blood sample was removed from the caudal vessels of each fish using a heparinized syringe. The blood was immediately transferred to a labelled, capped, polypropylene tube containing aprotinin (2 TIU ml^{-1} blood in 10 µl distilled water) on ice. The fish was killed by a blow to the head, placed in a labelled polythene bag and stored on ice in an insulated container.

After completion of sampling at each site the fish were transferred to the laboratory facility at the Hawick STW site where they were weighed, measured, and sexed. Scales were removed

from the flank of each fish with scalpel and forceps for age determination and placed in labelled envelopes. Gonadal tissue was dissected from the fish, weighed, and transferred to Bouins fixative. After 6 h the Bouins-fixed tissue was transferred to 70% methanol for storage until histological processing.

Blood samples were centrifuged (1 min) in an unrefrigerated benchtop centrifuge (Eppendorf) and plasma was removed by pipette, transferred to a labelled capped polypropylene tube and stored on dry ice in an insulated container for transfer back to CEH Windermere.

2.3.2 Northern Ireland

Wild brown trout were sampled by electrofishing at three sites (tributaries of the Lower Lagan) and on the dates shown in Table 3. Immediately after capture, fish were transported to the DANI Agricultural and Environmental Science Division Fish Laboratory in river water, rendered unconscious via immersion in an overdose of anaesthetic (2-phenoxyethanol), and killed by a sharp blow to the head. Fish were weighed and measured. Internal organs were exposed via a ventral incision and removed to reveal the gonads. Gonads were macroscopically sexed, removed intact and immersed in Bouins fixative. Livers were also removed and weighed before placing a transverse section 5 mm thick in Bouins fixative. After an initial period of 6 hours gonad and liver samples were transferred to 70% alcohol for storage until histological processing.

Table 3. Summary of sampling programme, Northern Ireland.

Site	Date	Number of fish sampled	
		Male	Female
Minnowburn (IGR J326685)	28/7/99	9	7
Collin River (Collin Glen) (IGR J275713)	3/9/99	9	3
Collin River (Dunmurry) (IGR J295693)	2/9/99	5	8
Ravernet River (IGR J288603)	14/8/99	4	9
Minnowburn River (IGRJ326685)	18/11/99	10	0
Totals		37	27

The Minnowburn and Collin rivers enter the Lagan downstream of the STW discharge and hence will draw breeding trout in late season from the affected reach. In this case, the Collin sample probably represents tributary resident fish. The Minnowburn was sampled twice – once in July yielding a sample of smaller, resident trout and once at spawning time when larger Lagan-derived spawning fish were captured. These fish, although captured near spawning sites in the Minnowburn, would almost certainly have been resident in the River Lagan itself in the in the summer months. Earlier electrofishing on 28/7/99 at the same site captured only small immature fish. It can therefore be assumed that these fish would have been exposed to the main River Lagan water with STW inputs prior to their pre-spawning entry into the tributary.

Data for the salmonid fry output on the River Lagan headwaters and the three lower tributaries (Ravernet, Collin and Minnowburn) are routinely collected on an annual basis as part of an ongoing salmon re-introduction project. As part of this exercise, trout fry are counted at a selection of sites using a standardised 5 minute electrofishing index (Crozier & Kennedy, 1994). The data for 1996-1999 were examined for general variation in abundance in order to assess whether there was any indication of a possible effect of the R. Lagan STW effluent discharge on trout production in the lower tributaries.

2.4 Tissue analysis

2.4.1 Gonadal histology

Histological procedures were carried out at the Environment Agency Brampton Fish Health Laboratory. To maximise the likelihood of detecting abnormalities within the gonadal tissue each gonad was divided into three equal portions, anterior, mid-portion and posterior. A centre section 3-5 mm thick from each portion was selected, giving a total of six transverse sections of tissue per fish. A Shandon Hypercentre XP tissue processor (with a 17 hour standard programme) was used to process the samples. All tissues were dehydrated through a graded alcohol series, cleared with chloroform and impregnated with paraffin wax before being wax blocked using a Shandon Histocentre 2 embedding centre. Tissues were sectioned with a base-sledge microtome at 3 μm, floated on a water bath at 45^{o}C and mounted on glass slides coated with Vectorbond reagent (Vector Laboratories Ltd., Peterborough.). Slides were stored at 50^{o}C for 24 hours before staining with Mayers haematoxylin and eosin. Three sections at 30 μm intervals were taken from each wax block. Slides were mounted with DPX mounting medium and examined microscopically. The stage of development, cell types and any structural abnormalities were recorded.

2.4.2 Measurement of vitellogenin concentrations in rainbow trout and brown trout plasmas

Assay of plasma VTG levels was carried out at CEFAS laboratories, Lowestoft. Separate reagents were used for the rainbow trout and brown trout vitellogenins (VTGs). Radioimmunoassays (RIAs) were used in both cases. However, the brown trout RIA used Atlantic salmon VTG as tracer and standard and a specific antiserum to brown trout VTG (a gift from Dr Birgitta Norberg). The rainbow trout RIA used entirely homologous reagents. The rainbow trout and salmon VTGs were prepared as follows:

Induction of VTG production: Powdered E_2 was added to melted cocoa butter at a concentration of 50 mg/ml. The powder was then evenly dispersed in the butter by ultrasonication (in a bath containing warm water) and injected intramuscularly into males at the rate of 400 μl (20 mg) per kg of fish. Blood was collected after an interval of 2 weeks.

Collection of blood: Prior to collection of blood samples, 2 ml syringes were rinsed with a saline solution containing 8 Trypsin Inhibitor Units (TIU)/ml of aprotinin and 500 IU/ml sodium heparin (to prevent blood clotting). Blood was transferred to 4 ml collection tubes, on ice, which contain 50 µl of heparin solution, with AEBSF and aprotinin at 1mg/ml and 8 TIU/ml, respectively. A maximum of 2.5 ml blood was added to each tube, spun at 2000 rpm and 4 ^{o}C for 30 minutes and the plasma removed, frozen and stored in liquid nitrogen.

Precipitation of VTG from plasma: Beforehand, the following solutions were made up and chilled on ice: 20 ml 20mM ethylenediaminetetraacetic acid (EDTA; adjusted to pH7.6 with 2M NaOH), containing 20 mg AEBSF; 2 ml 0.5M $MgCl_2$, containing 20 mg AEBSF; 100 ml distilled water, containing 36 mg AEBSF; 4 ml 1M NaCl in 50 mM Tris(hydroxymethyl)aminomethane (Tris) pH 8.0, containing 20 mg AEBSF and 0.8 TIU aprotinin; 20 ml 50 mM Tris pH 8.0, containing 20 mg AEBSF and 3.2 TIU aprotinin. Plasma samples from two E_2-injected male fish were thawed and dispensed as 1 ml aliquots into 150 mm x 16 mm glass tubes. Each tube then received 150 µl of the $MgCl_2$ solution, 3 ml of the EDTA solution and 15 ml distilled water. This produced a heavy white precipitate. The precipitate was compacted by centrifugation, the supernatant discarded, washed with 3 ml distilled water and compacted again.

Chromatographic purification: The precipitates from all tubes were redissolved, pooled in a maximum of 600 µl of the Tris/NaCl solution and then slowly made up to *c*. 20 ml with the Tris buffer. This solution was then injected through a 43 micron filter to remove particulate matter and loaded on to a prepared DEAE-Sephacel column (1 cm i.d. x 26 cm) at a rate of 0.5 ml/min, and at a temperature of 4 ^{o}C. The column was developed with a gradient formed by two pumps. Pump A reservoir contained 50 mM Tris pH8.0 and pump B reservoir contained 50 mM Tris, 1 M NaCl pH 8.0. Both buffers also contained 0.16 TIU/ml of aprotinin. The flow rate was 0.5 ml per minute. After the sample was loaded, the column was run with 5% B for 40 minutes and then a gradient of 5% B to 35% B over 200 minutes. Four minute fractions were collected. The effluent was monitored with a UV detector. Material which appeared in the wash was discarded. A major UV-absorbing peak eluted at about 150 minutes (corresponding to about 200 mM NaCl). The fractions around the peak were pooled, sealed in dialysis tubing and dialysed overnight against 5 litres of distilled water. The liquid

was then rapidly frozen in a flask of liquid nitrogen and freeze-dried.

Antiserum production: Antibodies were produced in rabbits by giving intramuscular injections of VTG (3 mg per injection per rabbit) dissolved in saline and emulsified with Freund's complete adjuvant. The rabbits were injected six times at two to three week intervals, and bled at least four times, at approximately monthly intervals.

Iodination: The salmonid VTGs were labelled with $Na^{125}I$ using Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) as the oxidising agent. The Iodogen was dissolved in dichloromethane at a concentration of 100 μg/ml; and 20 μl was allowed to dry in the bottom of a 1.5 ml Eppendorf vial. The vial was placed on its side. Freshly weighed VTG was made up in distilled water at a concentration of 1 mg/ml; and 10 μl was mixed with 20 μl of 0.5 M sodium phosphate buffer (pH 7.4) and pipetted onto the side of the vial. Ten μl of $Na^{125}I$ (3.7 MBq of radioactivity) was also pipetted onto the side of vial, which was then placed upright - allowing both solutions to run down into the base of the vial, where they came into contact with the Iodogen. The reactions was allowed to proceed for 10 min and then terminated by the addition of 1 ml 0.05M sodium phosphate buffer (pH7.4). This was added to 1.5 ml of assay buffer (see below), and applied to a prepared PD10 column (which contained Sephadex G25). The eluate from the column (2.5 ml) was allowed to go to waste. The radiolabelled protein fraction was then eluted by the addition of a further 3.5 ml assay buffer and collected in a glass vial containing 3.5 ml glycerol (to prevent the mixture freezing) and stored at -20 ^{o}C. Unattached $N^{125}I$ remained on the column.

Standard: About 1 mg VTG powder was weighed out and dissolved in distilled water at a concentration of 1 mg/ml. This was diluted 100-fold with assay buffer to form a solution with a concentration of 10 μg/ml.

Basic assay procedure: Plastic tubes were set up in trays - those which received the plasma samples (the 'unknowns') were labelled 1 to 60 in duplicate; those which received the standards were labelled S1 to S11 in duplicate. Three pairs of tubes were labelled M, B and T. Aliquots of 50 μl assay buffer were dispensed into all the sample tubes and 100 μl into S1 to S11, M and B. The volume in the sample tubes was made up to 100 μl by adding 50 μl of plasma in a neat or pre-diluted form. The diluted standard (100 μl) was added to each of the

S1 tubes. The tubes were mixed and 100 µl transferred to each of the S2 tubes. This procedure was repeated up till S11, when the final 100 µl was discarded. This yielded a standard curve with concentrations ranging from 5000 to 5 ng/ml. Anti-VTG serum was diluted 1:100,000 with assay buffer; and 100 µl was added to all tubes except for those labelled B and T. The tubes labelled B receive 100 µl assay buffer. After mixing, the tubes were centrifuged briefly to remove any drops on the sides of the tubes. They were returned to the rack, covered with aluminium foil and placed in the fridge overnight. The next morning, ^{125}I-VTG was diluted with assay buffer and 50 µl (20,000 cpm) added to all tubes. After another brief centrifugation, the tubes were incubated in the fridge overnight . To separate the bound from the free radiolabel, 100 µl of Sac-Cel (second antibody covalently linked to cellulose) was added to all tubes except for those labelled T. After a further 30 min, 1 ml distilled water was added to all tubes except those labelled T. The tubes were then centrifuged for 10 min to compact the cellulose, the aqueous phase removed by aspiration and the radioactivity adhering to the pellets measured with an automatic gamma counter. The T tubes represented the 'total' radiolabel added to each tube. The B tubes represent the amount of radiolabel bound to Sac-Cel in the absence of any anti-VTG antibody (i.e. non-specific binding). The M tubes represented the amount of radiolabel bound to anti-VTG antibody in the absence of any unlabelled VTG (i.e. maximum binding).

Using a spreadsheet, the activities in the standard and sample tubes were converted to 'percent bound' by dividing them by the total counts. When the percent of label bound was plotted against the logarithm of the VTG concentration, it formed a sigmoid curve. Unknowns were calculated from the steep middle portion of the curve. The only exception was samples with very low concentrations, which had to be calculated from the top part of the standard curve. The limit of detection of both assays (using 50 µl of 'neat' plasma) was 10 ng/ml.

Materials:

For purification of VTG:

Equipment: ultrasonic bath; FPLC dual pump chromatography system (with fraction collector and UV monitor)[1]; liquid nitrogen container; freeze drier; centrifuge.

Disposables: glass tubes (150 mm x 16 mm; carrying out precipitations); Acrocap filter (0.45 µm)[2]; HiTrap Q (1 ml) column[1]; PD-10 column (containing Sephadex G-25 M)[1]; dialysis tubing.

Chemicals: 17β-oestradiol; cocoa butter; aprotinin; 4-(2-aminoethyl)-benzene-sulphonyl

fluoride; ethylenediaminetetraacetic acid; sodium hydroxide; magnesium chloride; 0.05 M Tris buffer (made from 0.97 g Trizma base and 6.61 g Trizma hydrochloride dissolved in 1 L distilled water [pH 8.0 at 5 ^{o}C]); sodium chloride; DEAE-Sephacel; Sepharose 6B

For iodination:

Equipment: radioactivity monitor.

Disposables: eppendorf tube; PD-10 column[1]; glass scintillation vial (20 ml) for storing label; lead pots; pipettes; pipette tips; gloves.

Chemicals: ^{125}Iodine[3]; 0.5 M sodium phosphate buffer (made from 115 g di-sodium hydrogen phosphate [anhydrous] and 29.6 g sodium di-hydrogen phosphate [dihydrated] dissolved in 2 L distilled water and stored frozen in 50 ml aliquots; pH 7.4); 0.05 M sodium phosphate buffer; dichloromethane; Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril); glycerol; assay buffer (see below).

For radioimmunoassay:

Equipment: standard laboratory centrifuge with buckets which can hold up to at least 144 assay tubes; automatic gamma counter; water suction pump (i.e one which is attached to a tap) for the aspiration of the 'free' radiolabel fraction following the second antibody separation; tube racks; pipettes (covering the range from 5 µl to 5 ml); multiple pipettor (for repetitive dispensing of reagents).

Disposables: polystyrene tubes (12 mm x 75 mm); pipette tips; polystyrene pots (for mixing up reagents).

Chemicals: assay buffer (10.2 g di-sodium hydrogen phosphate [andydrous], 3.87 g sodium di-hydrogen phosphate [monohydrate], 8.18 g sodium chloride, 2.01 g potassium chloride, 500 µl Tween-20 and 1 g bovine serum albumin dissolved in 1 L distilled water; pH 7.2); Sac-Cel (second antibody covalently coupled to cellulose)[4]; radioiodinated VTG (stored at -20 ^{o}C; anti-VTG serum (stored at 4 ^{o}C at a dilution of 1:100 in 0.05 M sodium phosphate buffer, plus a few grains of sodium azide to prevent bacterial growth).

All chemicals and disposables were normally obtained from Sigma Chemical, Fancy Road, Poole, Dorset, BH12 4XA, UK except for: [1] - Amersham Pharmacia Biotech, 23, Grosvenor Road, St. Albans, Herts, AL1 3AW, UK; [2] - Gelman Sciences Ltd, Brackmills Business Park,

Caswell Road, Northampton NN4 7EZ, UK; [3] - Amersham Life Science Ltd, Amersham Place, Little Chalfont, Bucks, HP7 9NA, UK; [4] - Immunodiagnostic Systems Ltd, Boldon Business Park, Tyne & Wear, NE35 9PD, UK.

Rainbow trout samples from the effluent exposure experiment were assayed at a 1:1 dilution. Samples with values >5 μg/ml were re-assayed at 1:10 or at 1:100 and 1:1000. Samples from the wild-caught trout were assayed at a dilution of 1:20. Reference samples included in the same assays from fish exposed to high levels of estrogenic contaminants gave values in the 1 to 30 mg/ml range.

2.5 Water chemistry

Water samples from the Hawick sampling sites and those in Northern Ireland were analysed by SEPA West (responsible scientist: Susan Struthers) for total alkylphenol ethoxylates (total APEs).

2.5.1 Scotland

During October 1998, 12 samples of water from Hawick Sewage Treatment Works, the Teviot Water and Slitrig Water were received at SEPA West Region Laboratory in East Kilbride and analysed for nonylphenol (NP) and alkylphenol ethoxylates (APEs). The samples were double extracted using 50.0 ml of dichloromethane, the extracts were evaporated and made up to 1.0 ml in acetonitrile. The resultant extracts were run on an HP1050 HPLC with a Hypersil APS column and an HP1046A fluorescence detector. Using gradient elution and a mobile phase comprising a mixture of methyl-tetra-butyl ether (MTBE) and acetonitrile/methanol, a run time of 30 mins and an excitation wavelength of 230 nm and emission wavelength of 302 nm it was possible to separate and identify from nonylphenol-4-ethoxylate units (NP4EtO) up to nonylphenol-17-ethoxylate units (NP17EtO). The resulting chromatograms were quantified using mixed standards containing NP2EtO, NP5EtO and NP12EtO in known quantities. Because the standards did not contain discrete peaks but a range of peaks which overlap it was necessary to quote results as total APEs. Every NPxEtO peak was summed and then compared to a summed standard peak, to give the most accurate assessment of the APE content of the sample.

2.5.2 Northern Ireland

Replicate 1 litre water samples were taken from several points at the study sites and sent to the SEPA West Laboratories for APE analyses. Water samples were collected in acid-rinsed 1 litre glass containers and delivered to the analytical laboratory by overnight carrier. These samples were analysed for total alkylphenol ethoxylates as described above (2.5.1).

3. RESULTS

3.1 Assessment of effluent estrogenicity

3.1.1 Use of caged trout as biomonitors of estrogen exposure: Northern Ireland

The levels of VTG in the blood of the caged fish, and control fish sampled directly from the supplier, are presented in Table 4. ANOVA revealed no evidence of significant differences in plasma VTG levels between sites at each time (P = 0.49, 0.20, 0.11 respectively). Highest mean VTG levels were detected in fish caged in the final effluent tanks at the New Holland STW. Here, mean plasma VTG levels increased 15-fold during the period of exposure from a mean of 1.63 to 25.4 μg ml^{-1}. However, only four fish from the total of sixteen survivors displayed a marked VTG response, accounting for the large variation about the mean for this group.

Table 4. Plasma VTG levels (μg ml^{-1}) in caged and control rainbow trout (Northern Ireland)

Site	Dates	Initial sample		Mid-Expt Sample		Final Sample	
		Mean (μg ml^{-1})	SD (n)	Mean (μg ml^{-1})	SD (n)	Mean (μg ml^{-1})	SD (n)
R. Lagan at Barbour Campbell Mill (Upstream of STW discharge)	1/11/99 to 24/11/99	1.01	1.49 (20)	0.15	0.15 (20)	0.16	0.18 (20)
New Holland STW (final effluent tanks)	19/1/00 to 9/2/00	1.63	2.33 (20)	2.45	6.06 (15)	25.4	77.7 (16)
R. Lagan at Lambeg (downstream of discharge)	1/11/99 to 24/11/99	0.77	0.83 (20)	0.6	1.85 (19)	0.72	2.48 (19)
R. Bush Salmon Station (Control)	28/10/99 to 23/11/99	2.79	8.45 (20)	0.181	0.15 (20)	0.146	0.13 (20)
Source fish farm stock (Controls)	1/11/99 and 19/1/00	2.2	3.51 (20)	ND	ND	0.75	0.21 (10)

No significant differences were evident in the length, weight, gonadosomatic index (GSI) or

hepatosomatic index (HSI) for fish from any test site.

3.1.2 Semi-static aquarium exposure of rainbow trout to STW effluent: Scotland

Experimental fish: The mean weight and length of the rainbow trout employed in Experiment 1 were 221 ± 5 g and 27.5 ± 0.2 cm respectively (mean ± SEM; n = 48) and in Experiment 2 were 373 ± 15 g and 31.9 ± 0.3 cm respectively (n = 32).

Male/female differences: In Expt. 1 there was no significant difference overall (P = 0.3, ANOVA) between VTG levels in male (0.71 ± 0.7 μg ml^{-1}) and female (1.9 ± 0.97 μg ml^{-1}) fish at the start of the experiment and therefore the data for the sexes were combined. In Expt 2 there remained no overall significant difference between male (0.72 ± 0.01 μg ml^{-1}) and female (8.6 ± 3.8 μg ml^{-1}) fish (P = 0.2, ANOVA). The higher (though not significantly so) mean value for the female group and high variability about the mean was accounted for by some females which displayed relatively high values (male range 0.01 – 6.0; median 0.043; female range 0.09 – 64.2; median 0.66).

Blood vitellogenin levels: VTG levels in blood samples collected from these fish at the start and end of the exposure period are provided in Table 5 (Experiment 1) and Table 6 (Experiment 2).

The VTG data for each Experiment were subjected to a two-way analysis of variance (ANOVA, Genstat 5, 4.1; Lawes Agricultural Trust) with time (start, finish) and treatment (effluent concentration) as factors and with replicate tanks (1,2) within treatments.

There were no significant differences between VTG levels in fish exposed to increasing concentrations of STW effluent in either experiment (Tables 5 & 6).

Table 5. Effluent exposure experiment 1. November 1998. Plasma VTG concentrations (μg ml^{-1}) in rainbow trout prior to (pre-exposure) and following (day 11) exposure to Hawick STW effluent at a range of dilutions. Each value is the mean ± SEM, n = 12.

	Effluent concentration (%)			
	0	**7.5**	**15**	**30**
Pre-exposure	1.54 ± 1.33	0.84 ± 0.44	1.65 ± 1.08	1.66 ± 1.26*
Day 11	0.19 ± 0.1	0.14 ± 0.03	0.14 ± 0.07	0.23 ± 0.12

* One exceptionally high value (62.1μg ml^{-1}) was removed from this group prior to statistical analysis.

Table 6. Effluent exposure experiment 2. May 1999. Plasma VTG concentrations (μg ml^{-1}) in rainbow trout prior to (pre-exposure) and following (day 14) exposure to Hawick STW effluent at a range of dilutions. Each value is the mean ± SEM, n = 8.

	Effluent concentration (%)			
	0	**33**	**66**	**100**
Pre-exposure	41.8 ± 21.4	46.7 ± 17.9	122.8 ± 71.5	25.9 ± 12.3
Day 14	15.7 ± 9.3	7.4 ± 4.2	0.5 ± 0.2	1.0 ± 0.8

Overall, the mean VTG levels on day 11 (Expt 1; 0.17 ± 0.04 μg ml^{-1}) and day 14 (Expt. 2; 6.3 ± 2.3 μg ml^{-1}) were significantly lower than pre-exposure levels (Expt. 1: 1.42 ± 0.53 μg ml^{-1}; Expt. 2: 59.3 ± 9.7 μg ml^{-1}; $P<0.001$). This decline in VTG levels probably arises in part because the fish were not fed (feed may contain traces of phytoestrogens) during the exposure period and may also be related to stress associated with confinement. Neither factor is likely to have confounded a potential response to estrogenic stimulation. Trout possess extensive sequestered energy stores which can be utilised over short periods of starvation. Furthermore, although prolonged stress can reduce blood VTG levels and alter the abundance of estrogen receptors in the liver (Pottinger & Pickering, 1990) reproductive activity, including vitellogenesis, is not completely suppressed even by periods of exposure to a severe stressor (Campbell *et al*., 1994; Contreras-Sanchez *et al*., 1998).

3.2 Survey of natural salmonid populations at the study sites

3.2.1 Somatic data

3.2.1.1 Somatic data: Scotland

Age structure of samples: The age distribution of fish caught at the Scottish sample sites is presented in Table 7.

Table 7. Numbers of individuals within each year class at each of three sampling sites for each of three samples (Scotland).

		Age distribution			
Date of sample	**Sample site**	**0+**	**1+**	**2+**	**3+**
November 1998	River Teviot	-	16	1	-
	Slitrig Water	1	22	2	-
	Wilson Burn	-	17	2	-
April 1999	River Teviot	14	5	1	-
	Slitrig Water	6	13	-	-
	Wilson Burn	2	13	2	1
September 1999	River Teviot	2	28	-	-
	Slitrig Water	-	26	3	1
	Ale Water	-	10	18	1

November 1998: All the fish retrieved at each site were brown trout (*Salmo trutta*), and with a few exceptions were aged 1+ (in their second full year). There was no significant difference, overall, in the weight or length of fish from each site (Tables 8 & 9) or in the back-calculated length at one year old (Table 11). However, the coefficient of condition of fish from the R. Teviot was significantly ($P<0.05$) greater than that of fish from the other two sites (Table 10).

Table 8. November 1998: Body weight (g) of 1+ years old brown trout from each sampling site (Scotland) [mean ± SEM (n)]. ANOVA revealed no significant differences between sites.

	Immature 1+		Mature 1+		All 1+
Site	**Male**	**Female**	**Male**	**Female**	
R. Teviot	23.7 ± 2.96 (3)	36.0 ± 9.6 (10)	28.7 ± 1.9 (3)	None	32.4 ± 6.0 (16)
Slitrig Water	25.9 ± 5.1 (10)	33.2 ± 3.8 (11)	52 ± 11 (2)	44 (1)	32.2 ± 3.17 (24)
Wilson Burn	36.1 ± 4.3 (7)	34.6 ± 3.1 (9)	66 ± 11 (2)	None	38.7 ± 3.34 (18)

Table 9. November 1998: Fork length (cm) of 1+ years old brown trout from each sampling site (Scotland) [mean ± SEM (n)]. ANOVA revealed no significant differences between sites.

	Immature 1+		Mature 1+		All 1+
Site	**Male**	**Female**	**Male**	**Female**	
R. Teviot	13.3 ± 0.6 (3)	14.2 ± 1.1 (10)	13.4 ± 0.3 (3)	None	13.9 ± 0.7 (16)
Slitrig Water	13.2 ± 0.9 (10)	14.5 ± 0.6 (11)	16.7 ± 1.1 (2)	44 (1)	14.2 ± 0.51 (24)
Wilson Burn	15.2 ± 0.6 (7)	14.98 ± 0.5 (9)	18.1 ± 0.6 (2)	None	15.4 ± 0.4 (18)

Table 10. November 1998: Coefficient of condition ($100.\text{weight}/\text{length}^3$) of 1+ years old brown trout from each sampling site (Scotland) [mean ± SEM (n)]. ANOVA revealed a significant difference between sites ($P<0.05$).

	Immature 1+		Mature 1+		All 1+
Site	**Male**	**Female**	**Male**	**Female**	
R. Teviot	0.99 ± 0.02 (3)	1.07 ± 0.03 (10)	1.18 ± 0.02 (3)	none	1.08 ± 0.03* (16)
Slitrig Water	1.01 ± 0.02 (10)	1.02 ± 0.01 (11)	1.1 ± 0.02 (2)	0.94 (1)	1.02 ± 0.01 (24)
Wilson Burn	0.999 ± 0.024 (7)	1.006 ± 0.02 (9)	1.1 ± 0.07 (2)	none	1.01 ± 0.02 (18)

*Significantly greater than corresponding values for Slitrig Water and Wilson Burn

Table 11. November 1998: Back-calculated length (cm) at age 1 derived from readings of scales removed from the fish recovered at three sampling sites (Scotland) [mean ± SEM (n)] . ANOVA revealed no significant differences between sites.

Site	Back-calculated length at age 1 year (cm)
R. Teviot	6.92 ± 1.23 (17)
Slitrig Water	7.16 ± 1.26 (26)
Wilson Burn	7.03 ± 0.9 (20)

April 1999: As for the preceding sample, all the fish retrieved at each site were brown trout. A wider range of ages was present, reflecting the time of year at which the sample took place and most fish were either in the first (0+) or second (1+) year (Table 7). The 1+ fish recovered from the R. Teviot site were significantly smaller than those from the other two sites (Tables 12 and 13) although no difference was apparent in the back-calculated length at 1 year (Table 15) and coefficients of condition were similar for fish from each site (Table 14). Overall the coefficients of condition were lower for this sample (April) than the preceding sample (November) probably reflecting loss of weight during the winter months.

Table 12. April 1999: Body weight (g) of 1+ years old brown trout from each sampling site (Scotland) [mean ± SEM (n)]. ANOVA revealed a significant difference between sites.

	Immature 1+		Mature 1+		All 1+
Site	**Male**	**Female**	**Male**	**Female**	
R. Teviot	14.3 ± 3.03 (3)	13.8 ± 0.5 (2)	None	None	14.1 ± 1.7* (5)
Slitrig Water	39.4 ± 5.9 (10)	16.8 ± 0.8 (3)	None	None	34.2 ± 5.2 (13)
Wilson Burn	24.7 ± 1.3 (7)	29.9 ± 5.0 (6)	None	None	27.1 ± 2.4 (13)

* significantly lower than corresponding values for Slitrig Water and Wilson Burn ($P<0.05$).

Table 13. April 1999: Fork length (cm) of 1+ years old brown trout from each sampling site (Scotland) [mean ± SEM (n)]. ANOVA revealed a significant difference between sites.

	Immature 1+		Mature 1+		All 1+
Site	**Male**	**Female**	**Male**	**Female**	
R. Teviot	11.3 ± 0.95 (3)	11.3 ± 0.15 (2)	None	None	11.3 ± 0.5* (5)
Slitrig Water	15.8 ± 0.9 (10)	11.8 ± 0.09 (3)	None	None	14.9 ± 0.8 (13)
Wilson Burn	13.5 ± 0.2 (7)	14.4 ± 0.9 (6)	None	None	13.9 ± 0.4 (13)

* significantly lower than corresponding values for Slitrig Water and Wilson Burn ($P<0.05$).

Table 14. April 1999: Coefficient of condition (100.weight/length^3) of 1+ years old brown trout from each sampling site (Scotland) [mean ± SEM (n)]. ANOVA showed no significant differences between sites.

	Immature 1+		Mature 1+		All 1+
Site	**Male**	**Female**	**Male**	**Female**	
R. Teviot	0.96 ± 0.03 (3)	0.97 ± 0.004 (2)	None	None	0.96 ± 0.02 (5)
Slitrig Water	0.93 ± 0.02 (10)	1.01 ± 0.03 (3)	None	None	0.95 ± 0.02 (13)
Wilson Burn	1.00 ± 0.04 (7)	0.96 ± 0.02 (6)	None	None	0.98 ± 0.03 (13)

Table 15. April 1999: Back-calculated length (cm) at age 1 derived from readings of scales removed from the fish recovered from three sampling sites (Scotland) [mean ± SEM (n)] . ANOVA found no significant differences between sites ($P = 0.8$).

Site	**Back-calculated length at age 1 year (cm)**
R. Teviot	9.7 ± 0.99 (3)
Slitrig Water	9.4 ± 0.31 (13)
Wilson Burn	9.3 ± 0.19 (14)

September 1999: At this sample time, all the fish retrieved from the R. Teviot were found to be juvenile Atlantic salmon. Those from the other two sites were brown trout. In contrast with the preceding samples, a high proportion of male fish sampled at this time were sexually mature. Macroscopic examination of the gonads at time of capture suggested that no mature females were present but subsequent histological examination suggested that several females were in fact displaying signs of ovarian recrudescence. A comparison of weight, length, coefficient of condition and length at age 1 was carried out between the sites although the presence of salmon at site 1 may render such a comparison of dubious value. There was a highly significant difference, overall, in the size of 1+ fish from the three sites with fish from the Slitrig being significantly heavier (P<0.05 and 0.01; Table 16) and longer (P<0.05 and 0.001; Table 17) than those from the other two sites. There were no significant differences between the size of fish from the Teviot and Ale water. The coefficient of condition of fish from the R. Teviot was significantly greater (P<0.001; Table 18) than that of fish from the other two sites. This may be a function of the different body morphology of trout and salmon. There was no difference in coefficient of condition between the fish from Slitrig and Ale water sites. The back-calculated length at age 1 was significantly greater in fish from the Slitrig water compared to fish from the other two sites (Table 19).

Table 16. September 1999: Body weight (g) of 1+ years old brown trout and salmon from each sampling site (Scotland) [mean ± SEM (n)]. ANOVA revealed a significant difference in weight between sites (P = 0.008) resolved as fish from Slitrig Water being larger than those from the other two sites.

	Immature 1+		**Mature 1+**		**All 1+**
Site	**Male**	**Female**	**Male**	**Female**	
R. Teviot[1]	29.2 (1)	37.6 ± 6.8 (12)	35.0 ± 1.9 (15)	None	35.1 ± 3.2 (28)*
Slitrig Water	34.5 ± 8.1 (4)	41.9 ± 3.9 (11)	57.2 ± 4.4 (11)	None	47.2 ± 3.2 (26)
Ale water	32.6 ± 3.2 (3)	30.6 ± 1.4 (4)	35.7 ± 2.9 (3)	None	31.4 ± 1.5 (10)**

[1]All fish retrieved from this site were salmon. * significantly different from Slitrig, P<0.05; ** P<0.001.

Table 17. September 1999: Fork length (cm) of 1+ years old brown trout and salmon from each sampling site (Scotland) [mean ± SEM (n)]. ANOVA revealed a significant difference between sites (P=0.0001) resolved as fish from the Slitrig being significantly larger than those from the other sites.

	Immature 1+		Mature 1+		All 1+
Site	**Male**	**Female**	**Male**	**Female**	
R. Teviot[1]	13.7 (1)	14.1 ± 0.6 (12)	13.9 ± 0.25 (15)	None	13.9 ± 0.25 (28)***
Slitrig Water	14.3 ± 1.09 (4)	15.4 ± 0.5 (11)	16.8 ± 0.4 (11)	None	15.8 ± 0.3 (26)
Ale Water	14.4 ± 0.5 (3)	14.0 ± 0.3 (4)	14.7 ± 0.4 (3)	None	14.1 ± 0.26 (10)*

[1]All fish retrieved from this site were salmon. *** significantly different from Slitrig, P<0.001; * P<0.05.

Table 18. September 1999: Coefficient of condition (100.weight/length^3) of 1+ years old brown trout and salmon from each sampling site (Scotland) [mean ± SEM (n)]. ANOVA revealed a significant difference between sites (P<0.001). This was resolved as a significantly higher coefficient of condition for fish from the R. Teviot than for those at the other two sites.

	Immature 1+		Mature 1+		All 1+
Site	**Male**	**Female**	**Male**	**Female**	
R. Teviot[1]	1.136 (1)	1.25 ± 0.02 (12)	1.29 ± 0.01 (15)	None	1.29 ± 0.01 (28)***
Slitrig Water	1.12 ± 0.02 (4)	1.12 ± 0.01 (11)	1.18 ± 0.02 (11)	None	1.14 ± 0.01 (26)
Ale Water	1.08 ± 0.01 (3)	1.12 ± 0.03 (4)	1.1 ± 0.04 (3)	None	1.11± 0.02 (10)

[1]All fish retrieved from this site were salmon. *** significantly different from both Slitrig and Ale values, P<0.001.

Table 19. September 1999: Back-calculated length (cm) at age 1 derived from readings of scales removed from the fish recovered from three sampling sites (Scotland) [mean ± SEM (n)]. ANOVA revealed a significantly greater length at 1 year old for fish from Slitrig water than fish from the other two sites ($P = 0.008$).

Site	Back-calculated length at age 1 year (cm)
R. Teviot*	9.15 ± 0.15 (28)
Slitrig Water	10.12 ± 0.22 (29)
Ale Water	9.48 ± 0.26 (29)

*All fish retrieved from this site were salmon

3.2.1.2 Somatic data: Northern Ireland

A summary of somatic data for the fish sampled at sites in Northern Ireland is presented in Table 20.

Table 20. Somatic data for fish sampled at three sites in Northern Ireland. Values are mean ∀ SEM. Minnowburn River sampled on two occasions.

Site	Date of sample	Length	Weight	K-factor	GSI	HSI	n
Minnowburn River	28/07/1999	16.9 ∀ 0.8	67.1 ∀ 10	1.25 ∀ 0.03	0.7 ∀ 0.15	1.6 ∀ 0.15	16
Collin River (Collin Glen)	03/09/1999	20.0 ∀ 1.2	105 ∀ 15	1.17 ∀ 0.02	3.85 ∀ 0.6	1.50 ∀ 0.2	12
Collin River (Dunmurry)	02/09/1999	18.9 ∀ 0.7	89.3 ∀ 8.8	1.25 ∀ 0.03	3.27 ∀ 0.6	1.4 ∀ 0.14	13
Ravernet River	14/08/1999	24.0 ∀ 1.1	185 ∀ 25	1.22 ∀ 0.08	5.2 ∀ 1.8	---	13
Minnowburn River	18/11/1999	21.9 ∀ 1.0	149 ∀ 19	1.30 ∀ 0.07	3.8 ∀ 0.2	1.4 ∀ 0.05	10

Between-site variation was greater than was observed for the Scottish samples. ANOVA identified significant variation between sites in length ($P<0.001$), weight ($P<0.001$) and GSI ($P<0.05$). There were no significant differences between sites in coefficient of condition (K factor) or HSI.

Wild trout production in areas of the Lagan Catchment with differing levels of exposure to effluent containing potential endocrine disrupting chemicals are presented in Table 21. Values are 0+ fry densities (mean no. per 5 minute sample) with standard deviation.

Table 21. Wild trout production in areas of the Lagan Catchment with differing levels of exposure to effluent containing potential endocrine disrupting chemicals.

Increasing order of susceptibility of adults to STW influence)))))

Tributary	**Upper Lagan**		**Ravernet**		**Collin**		**Minnowburn**	
Year	**No. Sites**	**Mean no. trout (SD)**	**No. Sites**	**Mean No. trout (SD)**	**No. Sites**	**Mean No. trout (SD)**	**No. Sites**	**Mean No. trout (SD)**
1996	14	11.6 (10.7)	25	5.8 (4.6)	16	7.1 (4.0)	10	11.2 (8.5)
1997	24	25.0 (17.9)	25	2.4 (2.16)	16	5.8 (4.4)	6	4.7 (3.3)
1998	10	5.6 (5.7)	8	0.8 (1.2)	11	1.8 (2.4)	6	11.7 (7.1)
1999	20	11.5 (10.0)	9	1.8 (1.9)	15	14.7 (7.9)	6	10.2 (3.3)

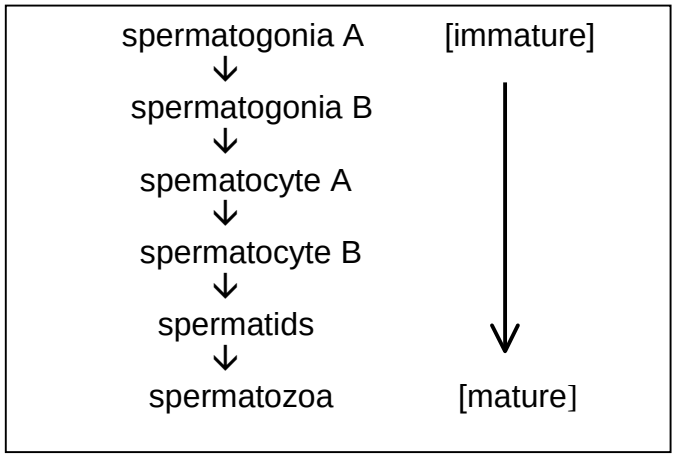
The comparison of 0+ trout densities for 1996 – 1999 for the feeder streams drawing broodstock from the affected lower reaches of the R. Lagan (Minnowburn and Collin), an interim site (Ravernet) and the cleaner headwaters some 40 km upstream, show no consistent pattern. The data suggest that is no evidence for direct effects of the R. Lagan STW effluent discharge on fry numbers.

3.2.2 Gonadal histology

The details of gametogenesis in salmonid fish are well described and several accounts have been drawn on for the description which follows (Nagahama, 1983; Lofts, 1987; Bromage & Cumaranatunga, 1988; Billard, 1992; Tyler & Sumpter, 1996; Pankhurst, 1998).

3.2.2.1 Salmonid gonadal morphology

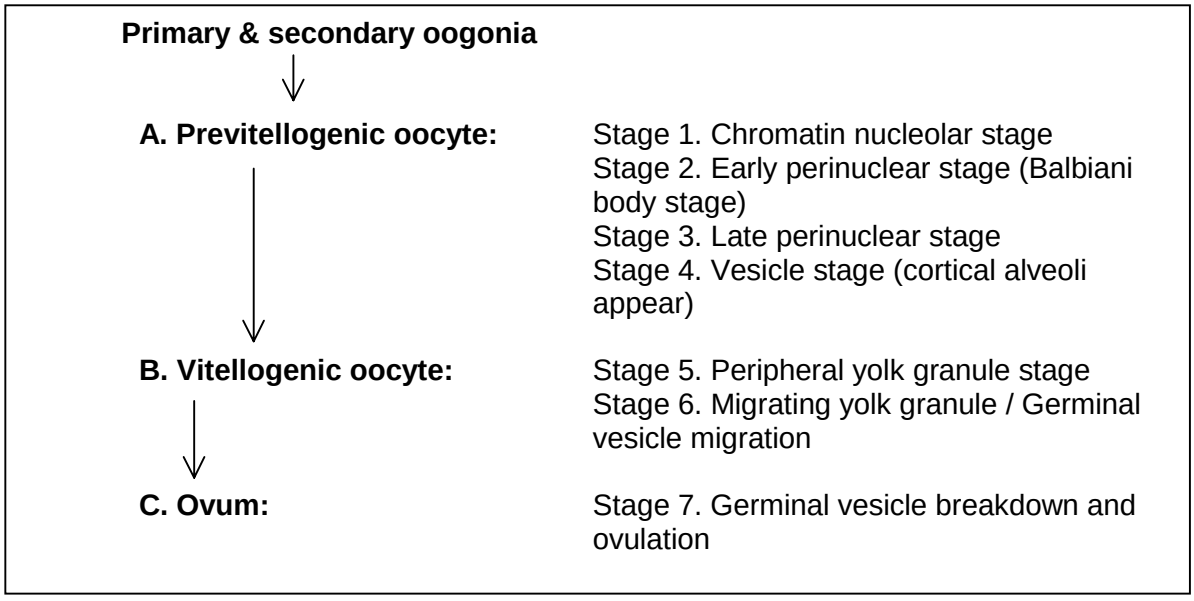
Males: In male trout and salmon, the testes are elongate paired organs sited adjacent to the dorsal body wall. The testis of salmonid fish are of the lobular type with sperm being released into the lobular lumen and from there into the sperm duct. In these fish spermatogenesis is cystic – primary spermatogonia undergo mitotic divisions to produce cysts containing several spermatogonial cells. The cysts are surrounded by somatic (Sertoli) cells. Spermatogonia divide mitotically to produce primary spermatocytes (spermatogenesis) which in turn divide meiotically to form secondary spermatocytes. The spermatocytes divide to give rise to spermatids and then spermatozoa (spermiogenesis). Spermatogenesis and then spermiogensis proceed in synchrony within the cysts until the cysts expand and ultimately rupture, liberating sperm into the lobular lumen and hence into the sperm duct (spermiation). For the purposes of the histological examination carried out during this study the developmental stages within the testes were characterised in the sequence:



Females: In salmonid fish the ovaries are also paired structures, lying longitudinally adjacent to the body wall. Brown/sea trout and Atlantic salmon possess group synchronous ovaries in which two populations of oocytes, at different stages of maturation, co-exist. In maturing females primary oocytes co-exist with larger oocytes destined to be spawned in the current season. The ovary consists of oocytes (ooplasm surrounded by the oolemma and zona radiata

i.e. the nascent egg) within somatic tissue (granulosa, theca and epithelium), together comprising the ovarian follicles.

The development of oocytes within the ovary can be subdivided into a number of characteristic stages. Primary germ cells are termed oogonia. These enlarge to form secondary oogonia. Oogonia persist throughout the life of the fish to provide for ovarian recruitment. Secondary oogonia are transformed into primary oocytes (oogenesis) coincident with the onset of meiosis. Oocyte development is subdivided into a number of readily identifiable stages with Stage 2 oocytes appearing in the ovary within 5 months of hatching and remaining most abundant until approximately 8 months after hatch. Stage 3 oocytes are present within 10 months of hatching and Stage 4 oocytes may be present within 12 months of hatch. During Stage 4 the zona radiata, or egg envelope, develops. During Stage 5 the oocyte increases dramatically in size as vitellogenesis is initiated and the yolk precursor vitellogenin is sequestered by the oocytes.



3.2.2.2 Examination of gonadal samples: Scotland

The critical feature being sought during the histological examinations of gonad material collected from the sampled fish was evidence for inappropriate presence of female-type gonadal tissue in male fish. This phenomenon (intersexuality) has been identified as being associated with long-term exposure to estrogenic contaminants in cyprinid fish in the UK and elsewhere.

Fixed, stained and sectioned gonadal tissue was examined from a total of 209 fish (R. Teviot: 65; Slitrig Water: 75; Wilson Burn / Ale Water: 69). No cases of intersexuality (female-type gonadal structure in testes samples) were evident in any of the sectioned material which was examined. A summary of the sex and maturity distribution for fish sampled at the Scottish sites is presented in Table 22.

Table 22. A summary of the numbers of fish within each sex and maturity category from each Scottish sample site on each of three sample dates.

Site	Date	Male		Female		Intersex
		Immature	Mature	Immature	Mature	
R. Teviot	18-19/11/98	2	3	11	0	0
Slitrig Water	18-19/11/98	9	4	11	2	0
Wilson Burn	18-19/11/98	5	6	8	0	0
R. Teviot	29/4/99	12	0	8	0	0
Slitrig Water	21/4/99	13	0	6	0	0
Wilson Burn	21/4/99	9	0	11	0	0
R. Teviot	6-7/9/99	0	15	14	0	0
Slitrig Water	6-7/9/99	4	14	10	2	0
Ale Water	6-7/9/99	5	15	4	6	0

November 1998: In the samples recovered during November 1998 the immature males displayed early stage spermatogonia (spermatogonia A) as the most abundant cell type with some spermatids and spermatozoa evident. The mature males were close to spawning or recovering from spawning with spermatozoa present in the sperm duct. In the immature female fish ovarian tissue was characterised by tightly packed perinuclear and balbiani body stage oocytes. Two mature females were found, both displaying early vitellogenic oocytes.

April 1999: During April 1999 all the sampled males displayed testicular tissue containing predominantly early stage spermatogonia (spermatogonia A). In some individuals some spermatozoa could be found in the centre of the lobules. All the sampled females were immature, characterised by tightly packed primary oocytes at the perinuclear and balbiani body stages with some evidence of early vitellogenic oocytes.

September 1999: In the September 1999 sample testicular tissue from mature male fish contained primarily spermatocytes (stages A and B) and spermatids. Some spermatogonia (stage B) were also evident. In the immature males the testes, at a macroscopic level, were thin and "stringy" in appearance. Microscopically, spermatogonia (stage A) were the major cell type. These were tightly packed with no lobular or tubular arrangement visible. The testes were well vascularised. Immature females were characterised by predominantly primary oocytes. Ovarian tissue from mature females contained large secondary (vitellogenic) oocytes leading to some problems with fixation and sectioning of the material. However, no evidence of abnormalities was evident. Some females displayed predominantly immature cell types (perinuclear and balbiani body stages) together with more advanced cortical alveoli stage and/or early vitellogenic secondary oocytes. In these fish there was some evidence of atresia suggesting that the decision to spawn in these individuals had been reversed.

3.2.2.3 Examination of gonadal samples: Northern Ireland

Minnowburn River, July 1999, November 1999: During July, mature males were most abundant among the sampled fish (n = 8). These fish were characterised by the presence of spermatozoa, but at an earlier stage of differentiation than males sampled later in the year. A high proportion of spermatogonia were present. The mature females present in this sample (n = 4) displayed mid- to late-vitellogenic oocytes with some Balbiani body, perinucleolar and cortical alveoli stage oocytes as well. A significant amount of atretic tissue was evident. Immature females were characterised by oocytes at the Balbiani body and perinucleolar stages.

During November only mature males were retrieved. These were all "running" (spermiated) characterized by large patches of spermatozoa in the duct tissue.

Collin River (Collin Glen), September 1999: This sample comprised mainly mature male fish (n = 8) all of which displayed proliferated duct tissue and spermatozoa together with spermatocytes and other cell types present in varying proportions. A single immature male displayed completely undifferentiated testes with only spermatogonia apparent. Two mature females displayed large late vitellogenic oocytes together with some atresia or reabsorption. The single immature female displayed a small number of early vitellogenic oocytes but predomintly Balbiani body and cortical alveolar stages.

Collin River (Dunmurray), September 1999: Equal numbers of immature and mature females (n = 4) were examined. Mature females displayed mainly late vitellogenic oocytes together with perinucleolar and Balbiani body stage oocytes. There was some evidence of atresia. Immature females were characterised by predominantly Balbiani body and perinucleolar stage oocytes. No immature males were present in the sample but mature males (n = 5) displayed spermatozoa adjacent to the duct tissue. Structure and cellular composition of the testes was normal.

Ravernet River (August 1999): A number of sampled females were mature (n = 5), characterised by large late vitellogenic oocytes together with some atresia. The immature females (n = 3) displayed Balbiani body, perinucleolar and early cortical alveoli stage oocytes. Mature males (n = 3) clearly showed spermatozoa, with some present in the duct tissue, together with spermatids and spermatocytes. Immature males (n = 2) were characterized by predominantly spermatogonia and generally undifferentiated testes.

3.2.3 Vitellogenin levels

3.2.3.1 Scotland

Plasma VTG levels were determined in blood samples from fish caught at the three study sites and are presented in Tables 23 to 25. Mean VTG levels were low throughout (< 2.0 μg ml^{-1}), the highest levels (30.5 μg ml^{-1}) being detected in the single mature female which was caught in Slitrig Water. All the data are presented combined in Table 26. An ANOVA of all the data, without partitioning by sex and after removal of the mature female, revealed a significant between-group difference (P = 0.01) which was resolved as higher VTG levels in the fish sampled from the R. Teviot compared to fish from the other sites (Table 26; P<0.01). This difference would appear to related primarily to higher levels among immature females recovered at this site; levels in the male fish taken from the Hawick site are as low, or lower, than males from the other sample sites. The very low levels of VTG throughout, and absence of elevated levels in male fish, suggest that the trout were not exposed to any estrogenic contaminants.

Table 23. Plasma VTG levels (μg ml^{-1}) in brown trout sampled from three sites during November 1998 (Scotland). Each value is the mean ± SEM (n). Maturity assessed by macroscopic inspection of gonads.

	Males	**Females**		**All fish**
Site	**Immature**	**Immature**	**Mature**	
R. Teviot	0.17 ± 0.06 (6)	1.87 ± 3.3 (11)	None	1.27 ± 2.7* (17)
Slitrig Water	0.14 ± 0.05 (14)	0.25 ± 0.2 (11)	30.5 (1)	0.19 ± 0.15** (25)
Wilson Burn	0.30 ± 0.63 (11)	0.27 ± 0.26 (9)	None	0.28 ± 0.49 (20)

*significantly different from the other two groups P<0.01

** mean excludes the value for the mature female

Table 24. Plasma VTG levels (μg ml^{-1}) in brown trout sampled from three sites during April 1999 (Scotland). Each value is the mean ± SEM (n). Maturity assessed by macroscopic inspection of gonads.

	Males	Females		All fish
Site	Immature	Immature	Mature	
R. Teviot	4.0 ± 3.7 (6)	7.1 ± 5.7 (8)	None	5.8 ± 3.5 (14)
Slitrig Water	<0.01 ND (13)	0.33 ± 0.15 (6)	None	0.11 ± 0.06 (19)
Wilson Burn	0.303 ± 0.12 (8)	33.3 ± 20.4 (11)	None	19.39 ± 11.9 (19)

Table 25. Plasma VTG levels (μg ml^{-1}) in brown trout (Slitrig Water and Ale Water) and Atlantic salmon (R. Teviot) sampled during September 1999. Each value is the mean ± SEM (n). Maturity assessed by macroscopic inspection of gonads.

	Males		Females		All fish
Site	Immature	Mature	Immature	Mature	
R. Teviot	0.08 (1)	0.19 ± 0.03 (15)	0.31 ± 0.04 (14)	None	0.24 ± 0.03 (30)
Slitrig Water	0.03 ± 0.01 (4)	1.7 ± 0.83 (14)	3.2 ± 2.68 (11)	14404 (1)	2.0 ± 1.08* (29)
Ale Water	0.08 ± 0.03 (5)	2.18 ± 1.56 (15)	10.9 ± 9.1 (9)	17850 (1)	4.53 ± 2.9* (29)

* Omitting values for mature females

Table 26. Summary of plasma VTG levels (μg ml^{-1}) in brown trout and Atlantic salmon sampled during November 1998, April 1999 and September 1999 from the sites in Scotland. Each value is the mean ± SEM (n). The value of *P* is derived from an analysis of variance and denotes the significance of site-to-site comparisons within each group. Maturity assessed by macroscopic inspection of gonads.

	Males			**Females**	**All fish**
Site	**Immature**	**Mature**	**All**	**Immature**	
R. Teviot	2.47 ± 2.25 (10)	0.19 ± 0.03 (18)	1.01 ± 0.8 (28)	2.47 ± 1.44 (33)	1.8 ± 0.9 (61)
Slitrig Water	0.06 ± 0.014 (27)	1.36 ± 0.65 (18)	0.58 ± 0.27 (45)	1.43 ± 1.06 (28)	1.3 ± 0.6 (74)
Wilson Burn Ale Water	0.17 ± 0.06 (20)	1.86 ± 1.24 (19)	0.99 ± 0.61 (39)	16.1 ± 8.4 (29)	7.4 ± 3.7 (68)
P	0.07	0.36	0.81	0.06	0.09

3.3 Water chemistry

3.3.1 Scotland

Water samples taken from the Hawick sites during October 1998 were analysed for alkylphenols and alkylphenol ethoxylates (Table 27). Very low levels of total alkylphenol equivalents were detected in the samples from Hawick (< 3.0 μg l^{-1}) (The proposed EQS for nonylphenol is 10 μg l^{-1}). Samples taken from the R. Teviot below Hawick, and from Slitrig Water detected total alkylphenol equivalents < 0.2 μg l^{-1}.

Table 27. Total APEs in water samples collected from the R. Teviot and Slitrig Water, during October 1998. F/E – final effluent.

Sample site and collection date	Total APEs (ug l^{-1})
Jackson Tool Hire (5/10/98)	<0.23
Slitrig Water at Hummelknowes Bridge (5/10/98)	<0.23
F/E Hawick STW (5-6/10/98)	1.02
F/E Hawick STW (7-8/10/98)	2.6
Teviot Water below Hawick (8/10/98)	<0.23
F/E Hawick STW (8-9/10/98)	0.69
F/E Hawick River sample (12/10/98)	0.75
F/E Hawick STW (12-13/10/98)	0.37
F/E Hawick STW (14-15/10/98)	0.2
Teviot Water below Hawick (15/10/98)	<0.23
Slitrig Water Hummelknowes Bridge (15/10/98)	<0.23
F/E Hawick STW (15-16/10/98)	0.56

3.3.2 Northern Ireland

Water samples taken from the Northern Ireland sites during 1999 were analysed for alkylphenols and alkylphenol ethoxylates (Table 28). Levels of total APEs were low at most sites (# 3 :g l^{-1}) with the exception of the sample retrieved from the Barbour Campbell Linen

Mill.

Table 28. Total APEs in water samples from Northern Ireland sites. November 1999, January/February 2000.

Sample site	**Date**	**Total APEs (:g l^{-1})**
Barbour Campbell Linen Mill u/s Control Site	29/11/99	12.2
New Holland STW d/s Site 1	29/11/99	1.62
Lambeg Fish Pass d/s Site 2	29/11/99	3.13
Stranmills Fish Pass d/d Site 3	29/11/99	2.1
R. Bush Control Site Salmon Station Bushmills	29/11/99	<0.24
Movanagher Fish Farm	25/1/00	<0.25
New Holland Waste WTW	1/2/00	1.76
New Holland Waste WTW	8/2/00	2.64

The concentration of APEs in the sample of Barbour Campbell Linen Mill has been confirmed by LC/MS analysis which was carried out in tandem with the fluorescence method and gives a result of 12.6 :g l^{-1}.

4. DISCUSSION

4.1 Estrogenicity of the STW effluents

Evaluation of the functional estrogenicity of the STW effluent at the Hawick and Lisburn sites was intended to be accomplished by siting cages containing immature rainbow trout above, immediately adjacent to, and downstream of the effluent discharge. This approach was successfully adopted in Northern Ireland but was unsuccessful in Scotland because of interference with the caged fish by members of the public. Instead, an aquarium-based exposure protocol was adopted to test the Hawick effluent.

On the R. Lagan in Lisburn trout exposed to the final effluent from New Holland STW displayed a pronounced elevation of blood vitellogenin (VTG) levels, from a mean starting value of approximately 2 :g ml^{-1} prior to exposure rising to a mean of 25 :g ml^{-1} after 3 weeks exposure. This is of a similar order of magnitude to the levels of VTG reported in rainbow trout exposed to effluent from the Ashford and Horsham STWs on the Rivers Stour and Arun respectively (Harries *et al*., 1997). However, the increase is extremely modest by comparison with the changes in blood VTG reported from similar studies conducted elsewhere. In rainbow trout caged at other STW sites in England and Wales blood VTG levels were elevated as high as 147,000 :g ml^{-1} and in the majority of cases exceeded 2,000 :g ml^{-1} (Purdom *et al*., 1994). Rainbow trout held in 100% final effluent from Harpenden and Chelmsford STWs displayed VTG levels in the range 10^7 – 10^8 :g ml^{-1} after 3 weeks exposure (Harries *et al*., 1999). In both these studies VTG levels in pre-exposure fish were of a similar order of magnitude to those in the present study. Therefore, although the New Holland effluent contains estrogenic components it must be assumed that their concentration is very much lower than is the case for some of the previously studied English STW effluents. Furthermore, most of the highly estrogenic effluents tested by Harries *et al*. (1999) were inactivated at a dilution of 25% suggesting that the New Holland effluent is unlikely to retain its activity when mixed with the main river.

No evidence of estrogenicity was obtained in the two trials which employed final effluent from the Hawick STW. In both aquarium-based trials blood VTG levels in effluent-exposed fish actually declined with time over the exposure period. This was particularly pronounced in

the second trial during May 1999 in which a number of maturing female fish were present resulting in mean blood VTG levels > 25 :g ml^{-1} at the start of the study. The significant decline in blood VTG levels which occurred in both the first and second trials may have arisen for a number of reasons including absence of feeding (direct nutritional effects or effects associated with the absence of phytoestrogens) and stress associated with the aquarium environment. We are confident that estrogenic activity would have been detected under these experimental conditions because a similar experimental design has been used at this laboratory to elicit vitellogenesis using water-borne estrogenics in both rainbow trout and chub (M. Juergens, T. G. Pottinger and N. Rajapakse, unpublished data).

The absence of estrogenic effects in fish exposed to 100% STW final effluent may appear surprising given the fact that published accounts of STW effluent estrogenicity emphasise the ubiquity of the vitellogenic response in exposed fish (Purdom *et al*., 1994; Folmar *et al*., 1996; Knudsen *et al*., 1997; Jobling *et al*., 1998; Harries *et al*., 1999). The final effluent from only one out of four STWs tested in the south east of England failed to elicit a vitellogenic response in caged trout (R. Stour, Bures STW; Harries *et al*., 1997) and this was suggested to possibly relate to the low population equivalent served by this site (1,238). However, recent work carried out under the COMPREHEND programme (Community Programme of Research on Environmental Hormones and Endocrine Disrupters, EC-funded, Year 1 Annual Report) has included a survey of STW effluent estrogenicity using caged trout in eight European countries. The results of these studies indicate that there is considerable variation in the estrogenic content of effluents with some sites registering no apparent functional estrogenicity. These preliminary data suggest that the greater the extent to which waste water is treated, the lower the levels of estrogenicity detected. A complete absence of estrogenic effects has also been reported for fathead minnows (*Pimephales promelas*) exposed to wastewater from representative central Michigan (U.S.A.) treatment plants (Nichols *et al*, 1999). The possibility of fluctuation in estrogenic activity over a prolonged period of time must also be considered, particularly where the final effluent may be subject to dilution by storm water influxes. Recent studies have suggested that short-term exposure of caged fish to STW effluent may not always detect estrogenicity where fluctuation in the concentration of estrogenic components occurs over time (Rodgers_Gray *et al*., 2000).

- **Conclusion:** Final effluent from New Holland STW contains estrogenic components but the elevation of blood VTG in exposed caged trout is low by comparison with many

English sites. Dilution of the effluent in the main river will further reduce its impact. Final effluent from Hawick STW was not found to be estrogenic

4.2 Vitellogenin levels in the sampled wild fish

Plasma VTG levels have been widely used as an indicator of exposure of wild (feral) fish to estrogenic contaminants. In studies on rivers in England, significant elevation of blood VTG levels has been detected as far as 5 km downstream of a point discharge of STW effluent (Marley STW, R. Aire; Harries *et al*., 1997). However, this was the exception and probably relates to additional industrial input; at three other sites, where estrogenicity of the effluent was probably related primarily to natural and synthetic steroids (Desbrow *et al*., 1998) estrogenic activity was detected only in the effluent itself (Harries *et al*., 1997). In the present study (excluding maturing females) no elevated VTG levels were found in any of the fish sampled from downstream of the Hawick STW, or at any of the other three sites from which fish were sampled.

Induction of detectable VTG production following exposure to an estrogenic stimulus is fairly rapid in rainbow trout, occurring within 3 to 7 days (Carragher & Sumpter, 1991; Pottinger & Rajapakse, unpublished data), but reportedly requires up to 10-15 days in brown trout (Sherry *et al*., 1999) although factors such as water temperature and dosage are important modulators of the induction process. Therefore, it is difficult to be specific regarding the period of time for which fish downstream of a point source input which is sufficiently concentrated to distribute an estrogenic signal into the main body of the river would require to have been resident for a response to be detectable. Furthermore, it is not known whether intermittent exposure to estrogens, such as might occur in fish moving upstream and downstream through an effluent plume, would have an effect on VTG induction. However, these points are academic as far as the Scottish survey data are concerned - the absence of evidence for elevated blood VTG levels in fish downstream of the Hawick STW is consistent with the aquarium exposure data which failed to detect any estrogenicity in the effluent.

The mean blood VTG levels reported for male and immature female fish from the Scottish sampling sites (–0.1 - 30 :g ml^{-1}) correspond with those reported for rainbow trout prior to

exposure to effluents (Harries *et al*., 1997; 1999) and also to levels reported for immature brown trout (Sherry *et al*., 1999). In contrast, blood VTG levels in maturing female brown trout which are actively vitellogenic can reach 100 mg ml^{-1} (Norberg *et al*., 1989) and in trout exposed to estrogenic effluent VTG levels of a similar order have been reported (Purdom *et al*., 1994). Two mature female trout recovered from the Slitrig water and Ale water during September 1999 displayed blood VTG levels of 14 – 18 mg ml^{-1} clearly indicating that the assay method was capable of detecting elevated VTG levels when present.

- **Conclusion:** No abnormally elevated levels of blood VTG were detected in any of the fish sampled from the R. Teviot or control sites on the Slitrig water, Wilson Burn and Ale water. There was no evidence that the sampled fish had been recently exposed to estrogenic contaminants.

4.3 Gonad structure in the sampled wild fish

A high incidence of abnormal gonadal structure has been reported for roach in English rivers (Jobling *et al*., 1998). These abnormalities were characterised by the simultaneous presence of both male and female gonadal tissue in fish which were macroscopically identified as male (intersexuality) and were present in 16% to 100% of fish caught downstream of STW inputs. However, a high incidence of intersexuality was also evident in fish captured upstream of STW inputs (12% - 44%) and in control populations (4% - 18%) (Jobling *et al*., 1998). Given that the numbers of intersex fish were inversely proportional to the numbers of fish with normal testes the authors concluded that the intersex condition was likely to represent feminization of male fish rather than masculinization of female fish. This interpretation was also consistent with the fact that many of the STW effluents in the study were known to contain estrogenic contaminants. It has been demonstrated that male carp (*Cyprinus carpio*) can be feminized by exposure to the estrogen mimic 4-*tert*-pentylphenol or to estradiol-17∃ (Gimeno *et al*., 1996). These observations, although their functional significance in terms of population level effects is as yet unknown, were a major impetus behind the present studies.

No evidence of intersexuality was detected in any of the gonad samples examined from sites in either Scotland (209 fish) or Northern Ireland (64 fish). All the samples from both male

and female fish presented a structural picture consistent with various stages of normal gonad development patterns. It is possible that this in part reflects the absence of strongly estrogenic effluents at either study sites. However, even were estrogenic contaminants present it is debatable whether salmonid fish would be susceptible to estrogen-induced intersexuality in the same way as is assumed to be the case for roach and other cyprinid fish.

The reproductive system of salmonid fish is believed to be most susceptible to interference by endocrine disrupting chemicals immediately prior to and during the period when sex differentiation is occurring. This coincides with gonadogenesis, which occurs very early in development and is the period during which the structural and supportive elements of the gonads are formed, and gametogenesis, during which formation of the gametes occurs (Hunter & Donaldson, 1983). Although the genotypic sex of the fish is determined by inheritance of specific genes, phenotypic sex can be modified because of the sensitivity of the gonadal tissue to endocrine factors. In rainbow trout, and presumably therefore other salmonid fish, sex differentiation occurs following yolk sac absorption and the onset of exogenous feeding. This fact is widely exploited to produce sex-reversed trout for aquaculture by manipulating the phenotypic sex of the fish using feed containing androgens or estrogens (depending on whether male or female fish are required) (Bye & Lincoln, 1986; Pandian & Sheela, 1995). Administration of estrogen to fish via the feed during this period can result in the production of 100% phenotypic females. In coho salmon (*Oncorhynchous kisutch*) it has been shown that a high frequency of the intersex condition can be induced by a single exposure (by immersion) of fish to estrogen (400 :g l^{-1}) between 8 days pre-hatch and 13 days post-hatch, prior to sexual differentiation taking place (Piferrer & Donaldson, 1989). In natural waters, resident salmonid fish are unlikely to be distributed downstream of STW or industrial inputs during this critically sensitive phase (pre-hatch - first feeding) and may therefore not be exposed to potentially feminizing compounds via this route. We are aware of no reports of intersexuality in wild salmonid fish populations which are similar to the condition described for roach (Jobling *et al*., 1998). There are only very isolated reports of intersex condition occurring at a macroscopic level in salmonids such as Arctic charr (*Salvelinus alpinus*; Fraser, 1997), schelly (*Coregonus lavaretus*; Brown & Scott, 1988) and Pacific salmon (*Oncorhynchus spp.*; Kinnison *et al*., 2000) and these are not thought to be related to contaminant exposure.

It is perhaps more likely that juvenile salmonid fish might be affected by estrogenic

contaminants from other sources, such as run-off from farmland to which animal waste has been applied. This waste has been shown to contain estrogenic steroids including estradiol-17∃ which appear in field run-off in measurable quantities (Nichols *et al*., 1997; Bushee *et al*., 1998). It is not clear to what extent agricultural practices in the areas in which salmonid nursery streams occur are likely to include spreading of animal waste. As already noted, no evidence of exposure to estrogenic contaminants was apparent in the present study. If the estrogenic contaminant levels were high enough in a STW effluent to sustain a significant concentration downstream of the effluent discharge, particularly at time of low river flow, it is possible that adult resident fish would be exposed. Whether this is likely to be of significance is unknown. For example, does maternal transfer of contaminants to the ovaries occur? Can short-term determinants of reproductive success (fecundity, sperm count, behaviour) be affected by exposure to contaminants? Some of these effects, were they to occur, might not necessarily be expected to be manifested as alterations in gonadal structure but might instead be reflected in population structure.

- **Conclusion:** Microscopic examination of gonadal structure in all the wild-caught fish revealed no evidence of abnormal developmental patterns or intersexuality. This may be a consequence of the absence of a significant estrogenic signal in effluent at either site but might also reflect the fact that salmonid gonadal development is most sensitive to estrogens at a period during which the fish inhabit relatively pristine nursery streams.

4.4 Water chemistry

A wide range of chemicals which enter the environment have been shown to have endocrine disrupting properties (Tyler *et al*., 1998). Two main classes of chemicals have been associated with estrogenic effects in exposed fish in UK waters. These are the alkylphenol polyethoxylates, which originate primarily from industrial sources but are also present in domestic waste, and steroids, which originate primarily from the human and animal population.

The alkylphenol polyethoxylates are broken down by microbial activity to alkylphenols (e.g. octylphenol, nonylphenol) which are known to be estrogen mimics (Nimrod & Benson, 1996). Steroids of human origin are excreted as inactive conjugates. These, too, are biodegraded to

release the active form of the molecule. The estrogenic steroid complement may contain a significant proportion of ethinyloestradiol (Larsson *et al*., 1999; Ternes *et al*., 1999), a potent estrogen employed in oral contraceptives, in addition to the natural estrogens, estrone and estradiol-17∃ (Belfroid *et al*., 1999; Kuch *et al*., 2000; Lagan *et al*., 2000).

Water samples from the R. Teviot and R. Lagan were not assayed for steroids. However, total alkylphenols (APs) and alkylphenol ethoxylates (APEs) were determined. Levels of APs and APEs from the Hawick sites were all low with highest levels being detected in final effluent from the STW of 2.6 :g l^{-1}. Levels of APs and APEs detected in final effluent from the New Holland STW on the R. Lagan were of a similar order (1.8 – 2.6 :g l^{-1}) but were notably higher in effluent from the Barbour Campbell Linen Mill (12.2 :g l^{-1}). Nonetheless, no evidence of a vitellogenic response was observed in the fish caged downstream of this effluent and these levels of APEs are considerably lower than reported for the heavily impacted R. Aire in Yorkshire (total extractable nonylphenol in effluent – 300 :g l^{-1} APEs; Harries *et al*., 1997). The only evidence of a vitellogenic response obtained in caged fish during these studies, fish exposed to New Holland STW effluent, is therefore more likely to be a consequence of the steroid content of the effluent than APEs.

- **Conclusion:** The chemical analyses suggest that neither of the study sites is exposed to a high level of APE contamination. The estrogenic effects detected in fish caged at the New Holland STW are likely to have arisen due to another component of the effluent, possibly steroids.

5. CONCLUSIONS

- Final effluent from New Holland STW contains estrogenic components but the elevation of blood VTG in exposed caged trout is low by comparison with many English sites. Dilution of the effluent in the main river will further reduce its impact. Final effluent from Hawick STW was not found to be estrogenic

- No abnormally elevated levels of blood VTG were detected in any of the fish sampled from the R. Teviot or control sites on the Slitrig water, Wilson Burn and Ale water. There was no evidence that the sampled fish had been recently exposed to estrogenic contaminants.

- Microscopic examination of gonadal structure in all the wild-caught fish revealed no evidence of abnormal developmental patterns or intersexuality. This may be a consequence of the absence of significant estrogenic contamination in effluent at either site but might also reflect the fact that salmonid gonadal development is most sensitive to estrogens at a period during which the fish inhabit relatively pristine nursery streams.

- The chemical analyses suggest that neither of the study sites is exposed to a high level of APE contamination. The estrogenic effects detected in fish caged at the New Holland STW are likely to have arisen due to another component of the effluent, possibly steroids.

Overall, these studies have produced no evidence that endocrine disruption of the reproductive system is occurring in salmonid fish in Scotland or Northern Ireland.

However, the relatively localised nature of these studies means that more widespread sampling of salmonid populations may be necessary to provide complete reassurance that salmonid populations are not being adversely affected by endocrine disrupting contaminants originating either from industrial, municipal or agricultural sources.

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